

Platelet-activating factor acetylhydrolase gene mutation in Japanese nephrotic children

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Background. Platelet-activating factor (PAF) may be involved in the pathogenesis of steroid-responsive nephrotic syndrome (SRNS). PAF is degraded to inactive products by PAF acetylhydrolase. We have investigated whether PAF acetylhydrolase gene mutation is involved in SRNS in Japanese children.

Methods. We identified a point mutation in the PAF acetylhydrolase gene (G994T) using the polymerase chain reaction in 101 Japanese children with SRNS and 100 healthy Japanese.

Results. There was no difference in the genotype and allele frequencies between patients with SRNS and normal controls. The mean number of relapses during the first year after onset was significantly higher in the 26 patients who were heterozygous for the mutant allele (GT) than in 75 wild-type homozygotes (GG) (2.61 ± 1.98 vs. 1.33 ± 1.35 ; $P = 0.0019$).

Conclusions. We conclude that analysis of the PAF acetylhydrolase gene mutation at position 994 in Japanese children with SRNS allows the identification of patients who are more likely to have a disease relapse.

Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid mediator that has been shown to have a variety of biological activities [1–3]. It appears to function both in normal physiological events and to mediate pathological responses, particularly allergy and inflammation. There is increasing evidence that PAF is involved in the pathogenesis of glomerulonephritis and nephrotic syndrome [4–7]. PAF is produced by inflammatory cells, endothelial cells, and glomerular mesangial cells [5, 6, 8, 9]. It possesses a multitude of biological effects pertinent to glomerular injury including chemotaxis and activation of leukocytes, complement activation, and con-

traction and stimulation of glomerular mesangial cells to produce prostaglandins and oxygen radicals [5, 10]. Furthermore, PAF also enhances vascular permeability and induces proteinuria [7, 10, 11].

The PAF concentration is tightly regulated at both the synthetic and degradative levels in order to avoid inappropriately high concentrations [1, 12, 13]. PAF is degraded to inactive products by hydrolysis of the acetyl group at the sn-2 position, to produce the biologically inactive products lyso-PAF and acetate. This reaction is catalyzed by PAF acetylhydrolase. Alterations in PAF acetylhydrolase activity have been reported in several disease states and may contribute to the pathogenesis of these conditions [14, 15].

Miwa et al reported an inherited form of PAF acetylhydrolase deficiency [16]. This autosomal recessive trait has only been observed in the Japanese population. Recently, Stafforini et al demonstrated that the PAF acetylhydrolase deficiency was the result of a point mutation near the active site of PAF acetylhydrolase (G to T transversion, at position 994 in exon 9) [17]. In patients who are homozygous for the mutation, enzymatic activity is completely abolished, while heterozygotes have reduced activity. An inherited deficiency of PAF acetylhydrolase activity has been associated with severe asthma in Japanese children [16]. Steroid-responsive nephrotic syndrome (SRNS) is often associated with allergic disorders [18]. We therefore postulated that the PAF acetylhydrolase gene mutation may play an important role in the pathogenesis of SRNS. In order to test this hypothesis, we investigated the effect of this mutation on the incidence of and relapse from SRNS.

METHODS

Patients. The following methods are in accordance with the ethical standards for human experimentation stipulated by the Ethics Committee of Kobe University Hospital. Informed consent was obtained from each individual.

Patients and controls

We studied 101 consecutive Japanese patients with SRNS, who visited 14 hospitals in Hyogo and Osaka

Key words: Japanese children, steroid-responsive nephrotic syndrome, relapse of renal disease, PAF, gene mutation, inflammation.

Received for publication April 3, 1998
and in revised form July 8, 1998

Accepted for publication July 9, 1998

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prefectures between January and September 1997. The age of onset of nephrotic syndrome in all the patients was less than 16 years. All the patients were followed for at least one year after disease onset. Thirty patients with frequent relapses underwent renal biopsy, and all of them showed minimal change nephropathy [19].

The definitions and criteria for nephrotic syndrome, remission, and relapse were the same as those used by the International Study of Kidney Disease in Children [19, 20]. Nephrotic syndrome was defined as urinary protein excretion ≥ 40 mg/hr/m² BSA with hypoalbuminemia (≤ 25 g/liter). Remission was defined as a reduction in the urinary excretion of protein to < 4 mg/hr/m² BSA (Albustix, 0 to trace) for three consecutive days. Relapse was denoted by the reappearance of proteinuria ≥ 40 mg/hr/m² (Albustix, ++ or greater) for three consecutive days.

Prednisolone was used as steroid therapy, the initial attack being treated with 2 mg/kg per day prednisolone, given in three divided doses (maximal dose 80 mg/day) for the first four weeks, followed by alternate-day prednisolone, with 1.3 mg/kg given as a single dose on the morning of every other day for four weeks (total 8 weeks). Relapses were treated with 2 mg/kg per day of prednisolone given in three divided doses (maximal dose 80 mg/day) for the first four weeks, followed by alternate-day prednisolone with 2 mg/kg given as a single dose on the morning of every other day for two weeks, after which dosage was decreased by 0.5 mg/kg every two weeks (total 12 weeks).

The patients' clinical data were collected retrospectively from the medical records. Patients for whom accurate data about the number of relapses were available were included in the present study.

One hundred unrelated Japanese adult healthy volunteers from the same ethnic group (age range 21 to 55 years), without any history of renal disease and having normal urine samples, were also studied as controls to determine the population frequency of the gene mutation.

DNA analysis

Genomic DNA was extracted and purified from peripheral leukocytes in whole blood samples with a SepaGene kit (Sanko, Tokyo, Japan). Polymerase chain reaction (PCR) was performed according to a modification of the method of Stafforini et al [17]. The genotype was determined by three independent amplifications. The sequences of the sense primer (Sense primer A) and three antisense primers (Antisense primer B, C, D) were as follows:

Sense primer A: 5'-CTATAAATTTATATCATGCTT-3'

Antisense primer B: 5'-TTTACTATTCTCTTGCTTTAC-3'

Antisense primer C: 5'-TCACTAAGAGTCTGAATAAC-3'

Antisense primer D: 5'-TCACTAAGAGTCTGAATAAA-3'

The PCR was performed in a final volume of 30 μ l, which included DNA (100 ng), 10 \times buffer (3 μ l), 50 mM MgCl₂ (1.0 μ l), 2.5 mM dNTP (2.35 μ l) and 2.5 units *Taq* polymerase (0.5 μ l) (Perkin Elmer, New Jersey, USA). The reac-

Table 1. Genotypes and allele frequencies of the platelet-activating factor acetylhydrolase gene in controls and patients with steroid-responsive nephrotic syndrome

	N	Genotype			Allele	
		GG	GT	TT	G	T
Controls	100	69 (69%)	30 (30%)	1 (1%)	0.84	0.16
Patients with steroid-responsive nephrotic syndrome	101	75 (74%)	26 (26%)	0	0.88	0.12

tions were performed as follows: (a) one cycle for 10 minutes at 94°C; (b) 5 cycles for one minute at 94°C, one minute at 56°C followed by one minute at 72°C; (c) 30 cycles for 30 seconds at 94°C, 30 seconds at 52°C followed by 30 seconds at 72°C; (d) one cycle for five minutes at 72°C. The sizes of PCR products were: 160 bp with primers A and B, and 108 bp otherwise. The products were analyzed by electrophoresis on 3% agarose gels stained with ethidium bromide.

The PCR products amplified by the sense primer A and antisense primer B correspond to the whole of exon 9 of the PAF acetylhydrolase gene. The PCR products amplified by the sense primer A and antisense primer C correspond to a part of exon 9 containing the normal sequence (G at position 994). The PCR products amplified by sense primer A and antisense primer D correspond to a part of exon 9 containing the mutation (T at position 994).

Assay for PAF acetylhydrolase activity

Plasma PAF acetylhydrolase activity was determined as described by Miwa et al [16].

Statistical analysis

The results were analyzed with StatView J-4.02 or SPSS 6.xJ software [21, 22]. The associations of categorical variables were examined by a chi-square test or Mantel-Haenszel test. Continuous characteristics of the two genotype groups were compared using the Mann-Whitney *U*-test. A two-tailed *P* value of less than 0.05 was considered to be significant.

RESULTS

Analysis of the genotype and allele frequencies of the PAF acetylhydrolase G994T gene mutation were performed in the patients with SRNS and the control population (Table 1). There was no significant difference in the genotype and allele frequencies between patients with SRNS and normal controls.

The clinical characteristics of patients with the GG genotype and GT genotype are summarized in Table 2. There was no significant difference between the two groups with respect to the sex ratio, mean age of onset and mean age at the time of study. However, the mean number of relapses during the first year after onset was significantly

Table 2. Clinical characteristics of patients with steroid-responsive nephrotic syndrome according to the genotype at position 994 of the platelet-activating factor acetylhydrolase gene

Genotype	GG (N = 75)	GT (N = 26)	P
Age at onset years	6.3 ± 3.7	6.1 ± 4.0	0.69
Age at study years	11.1 ± 4.6	11.1 ± 4.2	0.85
M/F	52/23	20/6	0.46
Plasma PAF acetylhydrolase activity (nmol/min/50 µl)	1.81 ± 0.61	0.88 ± 0.51	< 0.0001
Number of relapses during the one year from onset	1.33 ± 1.35	2.61 ± 1.98	0.0019

Data are mean ± SD except M/F.

Table 3. Number of relapses during the first year after onset according to the genotype at position-994 of the platelet-activating factor acetylhydrolase gene

		Number of relapses during the one year from onset				
		0	1	2	3	≥4
Genotype GG	25 (33%)	25 (33%)	8 (11%)	11 (15%)	6 (8%)	
Genotype GT	4 (15%)	4 (15%)	6 (23%)	3 (12%)	9 (35%)	

$\chi^2 = 10.3$, $p = 0.0012$ between genotype GG and GT groups.

higher in patients with the GT genotype than in those with the GG genotype (Mann-Whitney *U*-test, $P = 0.0019$). The mean plasma PAF acetylhydrolase activity was significantly lower in patients with the GT genotype than in those with the GG genotype (Mann-Whitney *U*-test, $P < 0.0001$).

We therefore examined the distribution of relapse during the first year after onset in patients with the GG genotype and GT genotype (Table 3). The results showed that patients with the mutant (GT) genotype had significantly more relapses than those with the wild-type (GG) genotype (Mantel-Haenszel test, $X^2 = 10.3$, $P = 0.0012$).

DISCUSSION

Studies in experimental animals indicate that PAF may also be an important mediator of renal damage [23–26], suggesting that its production and action in the kidney may be regulated in disease states. It has been shown that blockade of PAF receptors prevents proteinuria and glomerular injury in rabbit nephrotoxic nephritis [25]. Preliminary studies have also been performed in human renal diseases to evaluate the role of PAF [27–31]. Urinary and plasma PAF was increased in children with SRNS and levels correlated with disease activity [27]. Urinary PAF was significantly higher in patients with membranous nephropathy, and was positively correlated with proteinuria [28]. PAF was increased in the urine of children with hemolytic uremic syndrome, and levels correlated with disease activity [29]. PAF receptor mRNA has been detected in the kidney, being most abundant in the glomerulus [32]. These results indicate that PAF has a potential pathological role in glomerular injury. PAF acetylhydrolase is widely distributed in plasma [12], blood cells and a variety

of tissues including the kidney [33]. It is also present in urine [34]. PAF acetylhydrolase activity reflects the degree of PAF-dependent reactions, and may also be a potential candidate as a mediator of renal disease.

Recently, Tjoelker et al isolated the cDNA for human plasma PAF acetylhydrolase and showed that the recombinant enzyme abolishes PAF's pathological actions [35]. They also showed that the inherited deficiency of PAF acetylhydrolase in Japanese is the result of a point mutation in exon 9 [17]. Patients with the mutant allele are likely to have low PAF acetylhydrolase levels with an accumulation of PAF. However, the functional significance of the mutation in the PAF acetylhydrolase gene has not yet been established in renal diseases.

It has been suggested that genetic factors may determine susceptibility to SRNS. An association between some major histocompatibility complex antigens and SRNS has been described [36]. In the present study, the frequency in normal controls of the GG, GT and TT genotypes for the PAF acetylhydrolase gene was 69%, 30% and 1%, respectively. These are similar to the frequency previously reported in a Japanese population study (GG 69%; GT 27%; TT 4%) [17]. There was no significant difference in the genotype frequency between patients with SRNS and normal controls. These results indicate that there is no association between the PAF acetylhydrolase gene mutation and the susceptibility to SRNS.

A characteristic feature of SRNS is its tendency to relapse, the rate of which has been estimated to be between 40 and 90%. We have treated relapses with a 12-week course of prednisolone during the first year after onset; this treatment for relapse is popular among Japanese pediatricians. We have used cyclophosphamide or cyclosporine as adjuncts to prednisolone for inducing longer remissions in children who have frequent relapses beyond the first year after initial onset of nephrotic syndrome. We therefore examined whether there was an association between the PAF acetylhydrolase genotype and relapse during the first year after the onset of nephrotic syndrome. Relapse is considered a relevant event when assessing the prognosis of SRNS. In the present study, the number of relapses defined by the criteria of the International Study of Kidney Disease in Children [19, 20] was significantly higher in patients with the GT genotype than in those with the GG genotype. Of the 75 patients with the GG genotype, 66% had either no or one relapse, with only 8% having four or more relapses. In contrast, only 30% of the 26 patients with the GT genotype had either no or one relapse, while 35% had four or more relapses. Weak evidence links SRNS with the major histocompatibility complex [37], but there is no marker that correlates the frequency of relapse with the major histocompatibility complex in Japanese children. Although there is an association between post-medication hypocortisolism and relapse [38], there are no reliable clinical predictors of the risk of subsequent relapse following the initial episode

of SRNS. Assuming that our patients with SRNS are representative of the whole population of Japanese children with SRNS, analysis of the PAF acetylhydrolase gene mutation in Japanese children with SRNS may allow identification of a group of patients with a predisposition to relapse. Moreover, it is possible that therapy with a PAF receptor antagonist may prevent relapses in patients with SRNS.

The pathogenesis of SRNS is unknown. Shalhoub hypothesized that T-cells release a factor(s) that damages the glomerular basement membrane [39]. Although Shalhoub's hypothesis has not yet been proven, there is strong support for the concept. For example, release of the lymphokine called vascular permeability factor, which is produced by activated lymphocytes from nephrotic subjects, when injected intradermally causes increased permeability of the vessels to macromolecules [40]. Recently Sirois and Edelman demonstrated that vascular permeability factor increases vascular permeability by inducing PAF synthesis [41]. Plasma PAF acetylhydrolase activity was significantly lower in patients with the GT genotype than in those with the GG genotype in the present study. It can therefore be postulated that PAF accumulation due to a PAF acetylhydrolase deficiency will increase the permeability of the glomerular basement membrane and permit relapse of proteinuria in SRNS patients.

In summary, we have demonstrated an association between the PAF acetylhydrolase gene mutation and relapse in SRNS. Our study suggests that PAF plays a significant role in predisposing to relapse of SRNS. Additionally, it also suggests that analysis of the PAF acetylhydrolase gene mutation in Japanese children with SRNS may allow the identification of a group of patients who have a genetic predisposition to relapse.

ACKNOWLEDGMENTS

We thank Drs. K. Noguchi, S. Matsuyama, M. Ninomiya, S. Kodama, Y. Yamada, F. Suehiro, E. Maeda, M. Aida, K. Kobayashi, K. Ota, A. Kameda, H. Hachiwaka, K. Kitagawa, T. Ootsuka, H. Negishi, K. Shimomiya, S. Kariya, K. Yamaoka and K. Nakanishi for their support. The authors are also grateful to Dr. Y. Kiyoshi, 2nd Department of Pathology, School of Medicine Fukuoka University for helpful advice.

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